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(54) Title: TEST FOR TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES

(57) Abstract: A method of detecting transmissible spongiform encephalopathies is described which involves (a) treating a sample of tissue, blood or a blood derivative from a test subject with alcohol and a detergent, (b) adding an agent which degrades normal prion protein (c) adding an agent which denatures abnormal prion protein, (d) adding a prion-specific antibody and (e) detecting binding of the antibody to the sample. Also described is a method of fixing abnormal prion protein to a substrate comprising (a) treating the protein or a sample suspected to contain the abnormal prion protein with alcohol and an anionic detergent and (b) adding a protease, in the presence of the substrate.

WO 02/057789 A2

Test for Transmissible Spongiform Encephalopathies

The present invention relates to a method of detecting Transmissible Spongiform Encephalopathies, and in particular to a test which can be conducted on
5 living or dead animals or humans to identify TSEs.

Background to the invention

Spongiform Encephalopathies are a group of degenerative neurological diseases.
10 There are a number of examples of Spongiform Encephalopathies including BSE (Bovine Spongiform Encephalopathy), Scrapie, Creutzfeldt-Jacob Disease (CJD) Gerstmann-Straussler-Scheinker Syndrome, Kuru, Transmissible Mink Encephalopathy, Chronic Wasting Disease of Mule Deer, Feline Spongiform Encephalopathies and other Spongiform Encephalopathies found in animals such as elk,
15 naya, greater kudu, gemsbok and tigers. It has also been reported that BSE can be transmitted under laboratory conditions to mice and pigs. This crossing of species barriers by the infective agent has led to increased concern that transfer to humans could occur.

20 Bovine spongiform Encephalopathy (BSE) is a degenerative brain disorder of cattle which is popularly known as "mad cow disease". It has a slow incubation period, up to four or five years with symptoms of progressive degeneration of the mental state in cows include loss of co-ordination and staggering gait, lack of interest in their surroundings, disinterest in feed and water, or unpredictable behaviour, including
25 aggressiveness. Affected cattle show symptoms when they are three to ten years old.

First identified in Great Britain in November 1986, over 100,000 cases have since been recorded there. Post mortems of affected cattle reveal a characteristic pattern of vacuolation in the brain tissue due to destruction of neural cells and the deposition of
30 unusual protein fibres, that give the brain a spongy (spongiform) texture. Similar spongiform diseases have been recognised in humans (for example, Creutzfeldt-Jakob disease or (CJD) for over a century and in sheep (scrapie) for over 200 years. The agent

thought to be responsible for Transmissible Spongiform Encephalopathies is an infective protein known as a prion. The prion is an infective particle comprising protein only and no nucleic acid, the presence of nucleic acid being required in the case of a conventional virus. In Scrapie in particular one protein known as prion protein or PrP^{sc} has been found to co-purify with infectivity and can produce a Scrapie-like condition in brain cell cultures from other animals, such as hamsters under laboratory conditions. PrP^{sc} is the only known component of the characteristic protein fibres deposited in the brain tissue of Scrapie-infected sheep. This protein, the PrP^{sc} appears to undergo a structural modification, whereas the term PrP^c is used in respect of the normal cellular counterpart of PrP^{sc}. The natural function of PrP^c is not known, but it appears likely that it has an essential structure or functional role in the organism.

Recycled animal tissue, which had been routinely fed to British dairy cows as a protein supplement has been identified as the source of the infection. It is believed that BSE was originally spread from sheep's brains infected with scrapie and that its spread was accidentally accelerated by the ingestion of brain tissue taken from cows that had become infected with BSE. Therefore, the British Government introduced compulsory destruction of suspect animals and their carcasses beginning in 1988. The feeding of animal tissue to cows was banned in Britain in July 1988.

20

Since the initial report of the disease, consumers have feared that it might be transferable to humans through milk or beef products, particularly since Kuru, a related disease is known to be spread by ritualistic cannibalism among New Guinea tribesmen. In late 1990, consumer concern over the transmission of BSE to humans triggered a collapse in the beef market. A similar scare struck Germany in mid-1994.

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In 1996 ten cases of a newly described type of fatal CJD (Variant CJD) were identified. The victims had distinct brain tissue symptoms, were all under the age of 42, and had no hereditary record of the disease. It has been suggested that the victims may have contacted the disease through contact with BSE-infected cattle before the eradication of suspected animals had taken effect. The identification of Variant CJD led

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to a dramatic drop in beef consumption in Britain, and the banning of British and in some instances Irish beef imports in various countries worldwide.

Therefore, for both veterinary and economic reasons, there is an urgent need to
5 provide a method for diagnosis and a diagnostic kit to detect infection with BSE, Scrapie, Variant CJD and other related spongiform Encephalopathies, in livestock, animal carcasses and meat generally, as well as in pharmaceuticals and humans.

Object of the invention

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It is thus an object of the present invention to provide a method of testing animals, pharmaceuticals and humans for the infective agent responsible for TSE. It is also an object to provide a multitissue test system which can be used on both blood or blood derived products and solid tissue. It is a further object that the method be rapid,
15 with the result being available in a matter of hours, and that it should be cheap, reliable and user friendly.

Until this time there has been no method of detecting TSE which could be conducted on blood or blood derivatives. Up to now the only method of determining
20 whether animals were suffering from TSE or human beings were suffering from a related disease was to carry out a pathological post mortem examination of brain tissue. Thus a test which could be tested on blood has the enormous advantage that it can be conducted on living animals or human beings. This gives rise to the possibility that infected animals could be removed from the population and slaughtered, in an attempt to
25 prevent the spread of infection to other animals in the population. It would also mean that infected human beings could be identified, with the possibility that medical treatment could be administered and a possible cure could be found for the disease. The test would also prevent the entry of infected meat into the human food chain, thus reducing the possibility of humans contracting variant CJD or other related diseases
30 which may be transmitted by eating infected meat. It would also restore consumer confidence in meat or meat-derived products, which would be advantageous to both the farming community and the meat industry in general.

It has now surprisingly been found that a test for TSEs can be carried out on blood or blood derived products. The test has the advantage that it can also be carried out on solid tissue samples.

5

Summary of the Invention

According to the present invention there is provided a method of detecting TSE comprising (a) treating a sample of tissue, blood or a blood derivative from a test subject with alcohol and a detergent, (b) adding an agent which degrades normal prion protein (c) adding an agent which denatures abnormal prion protein, (d) adding a prion - specific antibody and (e) detecting binding of the antibody to the sample.

The invention also provides a method of fixing abnormal prion protein to a substrate comprising (a) treating the protein or a sample suspected to contain the abnormal prion protein with alcohol and an anionic detergent and (b) adding a protease, in the presence of the substrate.

Step (a) may further comprise the addition of a protein, preferably a high molecular weight protein such as bovine serum albumin, ovalbumin, normal serum of animal origin, an albumin substitute such as Prionex (trade mark), derivatives of such or the like. The protein may be formulated in a buffer containing up to 5%, preferably 0.05 to 0.4% protein.

Preferably the alcohol used in step (a) is a C₁ to C₂₄ alcohol such as methanol, ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, nonanol, decanol, undecanol, dodecanol, tridecanol, tetradecanol, pentadecanol, cetyl alcohol, heptadecanol, stearyl alcohol, nonadecanol, eicosanol, docasanol, dodecyl alcohol, lauryl alcohol, octadecanol, and derivatives of each, including primary, secondary and tertiary derivatives, or the like. More preferably the alcohol is a C₁ to C₅ alcohol, most preferably methanol/ethanol. The alcohol may be formulated in a buffer containing up to 30% (by weight), preferably 10 to 20% more preferably 8 to 12% alcohol.

Preferably the detergent of step (a) is selected from sodium dodecyl sulphate, sarcosyl, lithium dodecyl sulphate, cholic acid, deoxycholic acid, Triton X-100, octanoic acid, Brij (trade mark) 35 solution, N-Cetyl-N,N,N-trimethylammoniumbromide, CHAPS (3-[(α -cholamidopropyl)-dimethylammonio]-propane-sulphonate), CHAPSO (3-[(α -cholamidopropyl)-dimethylammonio]-hydroxypropane-sulphonate), Digitonin, nonanoyl-N-methylglucamide, n-Octyl-B-D-glucopyranoside, alkyl sodium sulphate, dioctyl sodium sulfosuccinate, sodium oleate, sodium lauryl sulphate, 1,2,3-Octanetriol, 3-Diethylamino-1 propyne, Chondroitinase ABC (available from Fluka), sodium Ionophore 1 (available from Fluka), and derivatives of each or the like, most preferably sodium dodecyl sulphate. The detergent may be formulated in a buffer containing 5 to 30%, more preferably 10 to 20% detergent. The detergent may be an anionic detergent.

In step (b) the agent which degrades normal prion protein may suitably be a protease, suitably a broad spectrum protease. Preferably the protease is selected from proteinase K, subtilisin Carlsberg, pepsin and derivatives of each or the like. The protease may be formulated in a buffer containing up to 10mg/ml, preferably 0.05mg/ml to 4mg/ml, more preferably 0.2mg/ml to 0.6mg/ml protein.

Preferably the agent which denatures abnormal prion protein in step (c) is a denaturing salt solution. Suitably the agent is selected from guanidine, urea, a standard denaturing solution e.g. Merck denaturing solution (commercially available from Merck) and derivatives of each or the like. In a particularly preferred embodiment the denaturing agent may further include up to 0.5M sodium hydroxide. The denaturing salt may be formulated in a buffer containing 0.1 to 10M, preferably 2 to 8M denaturing salt.

The buffer of step (a) (homogenising buffer) may be made up to have a final pH of 5.0 to 11.0, preferably pH 7.0 to 9.0, more preferably pH 7.5 to 8.5. The buffer of step (b) (differential buffer) may be made up to a final pH of 2.0 to 11.0, preferably pH 2.0 to 8.5, more preferably 7.5 to 8.5. The buffer of step (c) (assay priming buffer) should have a pH of 1 to 14, more preferably pH 9 to 12, most preferably at least 11.

Preferably, following step (b) the reaction mixture is washed with distilled water or a first wash buffer comprising up to 10%, preferably 4 to 6% buffered solution.

The assay priming buffer is allowed to incubate with the reaction mixture for about 5 to 30 minutes, preferably 10 to 20 minutes. Suitably, the mixture is then washed with a second wash buffer having a final pH of 5.0 to 11.0, preferably pH 7.0 to 8.0.

The first wash buffer may comprise sodium chloride, calcium chloride, zinc chloride or the like. The second wash buffer may comprise phosphate buffered saline or the like with the optional addition of 0 to 5% Tween 20 or the like.

Preferably the blood or tissue sample is dispensed onto a support, suitably a microtitre tray, and may then preferably be subjected to an optional centrifuge step which serves to clarify or "clean up" the samples for further processing.

The prion specific antibody is raised to a prion sequence which is to the 3' side of amino-acid number 60 of the prion sequence.

The prion specific antibody is suitably directed against the prion peptide:-

PRION SEQUENCE N-TERM

MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQ-44

GSPGGNRYPQGGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQP-87

GGGGWGQGGSHSQWNKPSKPPKTNMKHVAGAAAAGAVVGGLGGY- 131

MLGSAMSSPLIHFGNDYEDRYTRENMYRYPNQVYYRPVDRYSNQNN-177

wherein the single letter amino acid code is as defined herein, as well as derivative and fragments of the sequences, including those that may include D-isomer amino acids as well be understood by those skilled in the art, modification of this sequence by substitution, deletion or insertion of amino acids could result in a peptide with the same activity.

The blood sample may either be a whole blood sample, or it may be a blood derived sample such as a serum sample or a buffycoat preparation which includes white blood cells and leucocytes. If the test is used on a solid tissue sample a sample of about 0.3g to 1.5g is used.

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Suitably the blood or tissue sample is homogenised. If the blood sample is gelatinous an enzyme degradation step may be included. The enzyme degradation step may include the addition of DNase, Rnase, a protease or a lipase.

10 The prion specific antibody may be raised to a variety of species - specific prion sequences in any species. The antibody may be for example raised in rabbits, and the immunoassay may involve the use of a secondary antibody, suitably a goat (species) anti-rabbit antibody conjugated with horseradish peroxidase.

15 The detection method is suitably an enhanced chemiluminescence assay, although colourimetric detection, fluorimetric detection or radioimmunoassay detection could also be used.

In a particularly preferred embodiment there is provided a test for Transmissible
20 Spongiform Encephalopathies comprising obtaining a sample of blood or a blood derivative or tissue from a test subject, reacting the tissue, blood or blood derivative sample with a prion specific antibody and detecting the binding of the antibody to the sample by standard immunoassay, wherein the tissue, blood or blood derivative sample is mixed prior to addition of the antibody with a first buffer comprising 0 to 30% of C₁
25 to C₅ alcohol, 5 to 30% sodium dodecyl sulphate, or 5 to 30% sodium dodecyl sulphate and 0 to 5% sarcosyl, and 0 to 5% bovine serum albumin made up to volume in about 0.1 M TRIS/HCL or phosphate buffer, the buffer having a final pH of 7.0 to 9.0.

Preferably the blood or tissue sample is dispensed onto a support, more
30 preferably an inert plastic support, suitably a microtitre tray, and a second buffer comprising 1 to 10 mg/ml of a broad spectrum protease enzyme in about 0.1 M TRIS/HCL or phosphate buffer with a final pH of 7.5 to 8.5, is added. The buffer is

suitably diluted 1 in 5 to give a working strength of 0.2 mg/ml to 2 mg/ml. The sample is then incubated, preferably for about 10 to 180 mins at about 37 - 70°C, more preferably at 60°C.

5 Following incubation, the support suitably is washed with a volume of a first wash buffer comprising 0 to 10% salt solution. Preferably, a third buffer comprising either 0.5 to 3M guanidine or 2 to 8M urea, and 0.1 to 0.2M sodium hydroxide made up in reverse osmosis distilled water with a pH of at least 11 at room temperature, is added to the support. Suitably, the support and third buffer are allowed to incubate for about
10 15 minutes and are washed with a wash buffer comprising 0.1M phosphate buffered saline and 0.5% Tween 20™.

The sample preparation is then complete and ready for immunoassay.

15 The invention also provides a test for Transmissible Spongiform Encephalopathies comprising obtaining a sample of blood or blood derivative or tissue from a test subject, reacting the tissue, blood or blood derivative sample with a prion specific antibody and detecting the binding of the antibody to the sample by standard immunoassay, wherein the blood or tissue sample is dispensed onto an inert support and
20 incubated with a buffer comprising up to 10 mg/ml, preferably 0.05mg/ml to 4mg/ml, more preferably 0.2mg/ml to 0.6mg/ml, of a broad spectrum protease enzyme in a buffer with a final pH of 2.0 to 11.0, preferably pH 2.0 to 8.5, more preferably 7.5 to 8.5, prior to reaction with the antibody. Preferably the inert support is centrifuged following dispense of the sample and before addition of the protease(differential buffer). The
25 protease may be formulated in a buffer comprising 0.1M TRIS or phosphate.

In a further aspect the invention provides a test for Transmissible Spongiform Encephalopathies comprising obtaining a sample of blood or a blood derivative or tissue from a test subject, reacting the tissue, blood or blood derivative sample with a prion
30 specific antibody and detecting the binding of the antibody to the sample by standard immunoassay, wherein the sample is incubated with a buffer comprising 0.1 to 10M denaturing solution, preferably 3 to 6M, more preferably 0.5 to 3M Guanidine (optimal

range 2 to 4M) or 2 to 8M urea (optimal range 4 to 6M), and up to 0.5M, preferably 0.1 to 0.2M sodium hydroxide made up in reverse osmosis distilled water, with a pH of at least 11 at room temperature, prior to reaction with the antibody.

5 The invention also provides a buffer selected from:

1. 0 to 30% of C₁ to C₂₄ alcohol (optimal range 10 to 20, preferably 8 to 12%) 5 to 30% detergent (optimal range 10 to 20%), and a 0 to 20% protein (optimal range 0.05 to 0.4%) made up to volume in a buffer having a final pH of 5.0 to 11.0 (optimal range 7.5 to 8.5).
- 10 2. A buffer comprising of 0.05mg/ml to 4mg/ml (optimal range 0.2mg/ml to 0.6mg/ml) of a protease enzyme in a buffer having a final pH of 2.0 to 11.0 (optimal range 2.0 to 8.5).
3. A buffer comprising 0.1 to 10M denaturing solution (optimal range 2 to 8M).

15 All buffers are produced and supplied by Enfer Scientific Ltd.

Homogenising Buffer:

Ingredients	Working Range	Optimal Concentration
Methanol or Ethanol	0 to 30%	16%
Sodium Dodecyl Sulphate	5 to 30%	15%
Bovine Serum Albumin	0 to 5%	0.1%

20 This buffer is made up to the required volume, stirring in 0.1M TRI/HCL Buffer, final pH of 8.0 \pm 1.

Differential Buffer:

Ingredients	Working Range	Optimal Concentration
Proteinase K	0.05 to 4mg/ml	0.4mg/ml

25 This buffer is made up to the required concentration in 0.1M TRIS/HCL Buffer, final pH of 8.0 \pm 1.

Assay Priming Buffer:

Ingredients	Working Range	Optimal Concentration
Guanidine	0.5 to 6M	3M
Sodium Hydroxide	0 to 0.5M	0.15M

OR

Ingredients	Working Range	Optimal Concentration
Urea	2 to 8M	6M
Sodium Hydroxide	0 to 0.5M	0.15M

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This buffer is made up to the required concentration in reverse osmosis distilled water and allowed to reach room temperature before checking the pH, which should be greater than 11.

10 **Wash Buffer1:**

Ingredients	Working Range	Optimal Concentration
Sodium Chloride	0 to 10%	5%

This buffer is made up to the required volume in reverse osmosis distilled water, pH irrelevant.

15 **Wash Buffer 2:**

Ingredients	Working Range	Optimal Concentration
Phosphate Buffered Saline	0 to 5M	0.1M
Tween 20	0 to 5%	0.5%

This buffer is made up to the required volume in reverse osmosis distilled water, with a pH of 7.6 ± 1 .

20

The invention further provides a buffer selected from:

1. 0 to 20% of C₂ to C₅ alcohol, 15 to 30% sodium dodecyl sulphate, or 5 to 30% sodium dodecyl sulphate and 0 to 5% Sarcosyl, 0 to 0.5% bovine serum albumin and made up to volume in about 0.1M TRIS or phosphate buffer, the buffer having a final pH of 7.0 to 9.0;
2. A buffer comprising 1 to 10 mg/ml of a broad spectrum protease enzyme in about 0.1M TRIS or phosphate buffer with a final pH of 7.5 to 8.5;
3. A buffer comprising either 0.5 to 3M Guanidine or 2 to 8M urea, and 0.1 to 0.2M sodium hydroxide made up in reverse osmosis distilled water, with a pH of at least 11 at room temperature.

The invention will now be described in greater detail with reference to the following example.

EXAMPLE

The requirements for the TSE immunoassay are as follows:

- a) A sample of solid tissue, blood or blood derived preparation.
 - b) A series of preparative sample treatments.
 - c) A prion specific antibody.
 - d) Sensitive method of detection.
- a) A sample of blood is taken using either an anti-coagulant coated tube or a non-coated blood tube. Two options are available for testing:
- i) Whole blood sample.
 - ii) A blood derived sample - serum sample or a buffycoat (white blood cells, leucocytes etc.) preparation.
- A sample of solid tissue is taken from an animal or human by a suitable method e.g. biopsy or dissection.
- b) On reaching the laboratory the sample is identified using barcodes. The barcode assigned to each sample incorporates information on the place of origin of the sample

(slaughter plant, clinic, hospital), the date the sample is taken and a traceable identification sample number. An amount of each sample, is removed, weighed and placed into a sample bottle/tube/stomacher bag for homogenisation (stomaching, sonication or vortexing). If the sample is particularly gelatinous, an enzymatic degradation step can be included - addition of DNase, Rnase, protease or lipase used in accordance with supplier instructions. This sample bottle or bag containing the homogenised original sample will be assigned an identical barcode to the original sample. This allows full traceability throughout the system. A fixed volume to weight ratio (1:1 to 1:25) of Homogenising Buffer, supplied by Enfer Products, Castleblake, Rosegreen, Cashel, Co. Tipperary, is added to the sample bottle and the sample homogenised by stomaching, vortexing or sonication. If the sample is particularly gelatinous, an enzymatic degradation step can be included -addition of DNase, Rnase, protease or lipase used in accordance with supplier instructions. The sample is then dispensed (200 μ l) in duplicate onto a polypropylene 96 well microtitre plate. Blank, Negative and Positive controls supplied by Veterinary Research Laboratories, Abbotstown, Dublin are also added to this plate. This plate is centrifuged in a plate centrifuging rotor at 4000 rpm for 10 minutes. 20ul of Differential Buffer, supplied by Enfer Scientific, Castleblake, Rosegreen, Cashel, Co. Tipperary, is added preferably to a plastic, totally inert microtitre plate (e.g. DYNEX Microlite 1 plate made from immulon 1 plastic). The plate has to be inert for optimal completion of the assay. Use of charged plates decreases the results. 100 μ l of each of the centrifuged samples and controls is then transferred from the polypropylene centrifuged plate to the inert assay microlite plate containing the volume of Differential Buffer and a plate coverslip placed over the wells. The plate is then incubated at 37°C for 60 minutes, the coverslip removed and the plate washed in Wash Buffer 1, supplied by Enfer Scientific, Castleblake, Rosegreen, Cashel, Co. Tipperary. Assay priming Buffer, supplied by Enfer Scientific, Castleblake, Rosegreen, Cashel Co. Tipperary, is added to each well at a volume of 200 μ l. The plate is covered with a coverslip and incubated at 37°C for 15 minutes. The coverslip is removed on completion of the incubation and washed with Wash Buffer 2, supplied by Enfer Scientific, Castleblake, Rosegreen, Cashel Co. Tipperary, is then added to the samples on the plate. This step completes the sample preparation procedure.

c) The samples are now ready for immunoassay. This requires a **prior** specific antibody. An example for such an antibody is a rabbit anti - PrP, raised by **injecting** rabbits with ovalbumin conjugated synthetic prion peptides, a peptide which is **licenced** to Enfer Scientific by Proteus International UK. This peptide was identified by Proteus International UK using molecular modelling techniques followed by Western Blot and ELISA analysis. The peptides are synthesised in bulk using solid-phase peptide synthesis. Use of other anti-PrP antibodies raised to a variety of species - specific prion sequences in any species, will also yield satisfactory results in this assay. The peptide described herein is only one example of an antibody that has been **successful** in the assay. Both polyclonal and monoclonal antibodies can be used for assay.

A fixed volume (150 μ l) of this specific antibody at optimal working strength dilution in Wash Buffer 2 is added to the microlite plate, incubated at 37°C for 40 minutes and washed using Wash Buffer 2. A fixed volume (150 μ l) of species-anti-rabbit(species)-HRP supplied by DAKO, USA at optimal working strength dilution in Wash Buffer 2 is added to the plate, incubated at 37°C for 30 minutes and washed using Wash Buffer 2. Detection of results is now possible.

d) Detection of results is by means of Enhanced Chemiluminescence. A fixed volume of a chemiluminescent reagent- Amerlite reagent (150 μ l) supplied by Johnson & Johnson, UK, is added to the microtitre plate and incubated at 37°C for 3 minutes. The light signal is read using a Labsystems Chemiluminometer, the results assigned to the corresponding barcode and a results report printed.

DETAILED PREPARATIVE PROTOCOL

25

1. HOMOGENISATION

1.1 Blood or blood derived or tissue sample is placed into a sample bottle/tube/bag.

1.2 1 to 25, preferably 10 to 15 times the sample weight of Homogenising Buffer is added to the sample.

30 1.3 This mixture is homogenised (stomacher, sonicator, vortex) for 2 minutes or until the sample is totally homogenised.

1.4 Enzymatic degradation option may be applied here at the discretion of the operator.

1.5 The resultant homogenate may be filtered, if particulate matter remains in the sample.

2. PLATING

- 5 2.1 A V-96-well microtitre polypropylene plate is used for centrifugation of 200 μ l of each sample.
- 2.2 200 μ l of blank control, onto the plate, positions A1,2 of the centrifuge plate.
- 2.3 200 μ l of negative control (known negative BSE homogenate), supplied by the Veterinary Research Laboratories, Abbotstown, Castleknock, Dublin, Ireland is
- 10 dispensed - 4 replicates- onto the plate, positions B1,2 and C1,2.
- 2.5 200 μ l of positive control, (known positive BSE homogenate), supplied by the Veterinary Research Laboratories, Abbotstown, Castleknock, Dublin, Ireland is
- dispensed - 4 replicates- onto the plate, positions D1,2 and E1,2.
- 2.6 200 μ l of each (filtered, enzymatically treated) homogenate is dispensed in
- 15 duplicate onto the plate, into the remaining positions.
- 2.7 The plate is centrifuged at 4000rpm for 10 minutes.
- 2.8 20 μ l of Differential Buffer is added to each well of an inert assay microtitre plate e.g. a DYNEX microlite 1 plate.
- 2.9 100 μ l of each centrifuged sample and control is removed from the centrifuge
- 20 plate and dispensed onto the same position on the inert assay microlite plate, bringing the well volume to 120 μ l.
- 2.10 The plate is covered with a microtitre plate sealer and incubated at 37°C for 60 minutes.
- 2.11 The plate is then washed 4 times with Wash Buffer 1.
- 25 2.11 200 μ l of Assay priming Buffer supplied by Enfer Products, Castleblake, Rosegreen Cashel, Co. Tipperary, Ireland, are added to each well of the microtitre plate and the plate incubated for 15 minutes at 37°C.
- 2.12 The plate is washed 4 times with Wash Buffer 2.

30 3. IMMUNOASSAY

- 3.1 150 μ l of primary antibody, rabbit (species) anti-PrP, polyclonal/monoclonal antibody at optimal dilution is dispensed onto the plate.

- 3.2 The plate is incubated at 37°C for 40 minutes.
- 3.3 The plate is washed 4 times with Wash Buffer 2.
- 3.4 150µl of secondary antibody, species-anti-rabbit(species)-HRP at optimal dilution is dispensed onto the plate and the plate incubated at 37°C for 30 minutes. Optimal
- 5 dilution is determined by a grid assay e.g. plotting varying concentrations of primary and secondary antibody to determine optimal light signal (see below).
- 3.5 The plate is washed 4 times with Wash Buffer 2.

4. DETECTION

- 10 4.1 150µl of an enhanced chemiluminescent reagent-Amerlite reagent, supplied by Johnson & Johnson Clinical Diagnostics, UK is added to the plate.
- 4.2 The plate is incubated at 37°C for 3 minutes.
- 4.3 The light signal is read using a Labsystems Chemiluminometer, supplied by Medical Supply Company, Dublin, Ireland, which reads the luminescence from each
- 15 well of the plate by scanning the entire IR-Visible-W spectra and extrapolating results.
- 4.4 The light signals from the plate are transferred to a customised software package, designed by G.K.S. Software, Dublin Ireland.
- 4.5 Each light signal is assigned to a corresponding bar-code and a report printed.
- 4.6 Results are quoted in chemiluminescent light units - L.U.

20

SOURCE OF REAGENTS

1. Prion Specific Antibodies - anti-PrP- are supplied by Enfer Scientific Ltd. and are polyclonal antibodies raised to the following synthetic prion peptide:

25 PRION SEQUENCE

N-TERM -

MVKSHIGSWILVLFVVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQ- 44

30 GSPGGNRYPPQGGGGWGQPHGGGGWGQPHGGGGWGQPHGGGGWGQ-87

GGGGWGQGGSHSQWNKPSKPPKTNMKHVAGAAAAGAVVGGLGGY- 131

MLGSAMSSPLIHFGNDYEDRYTRENMYRYPNQVYYRVPVD RYSNQNN-177

Single Letter Amino Acid Code:

A -Alanine, C- Cysteine, D -AsparticAcid, E- GlutamicAcid, F- Phenylalanine, G-
5 Glycine, H - Histidine, I - Isoleucine, K - Lysine, L - Leucine, M- Methionine,
N-Asparagine, P - Proline, Q - Glutamine, R -Arginine, S- Serine, T- Threonine, V-
Valine, W- Tryptophan, Y- Tyrosine.

This 29 amino acid peptide is used to satisfactorily raise anti-PrP antibodies for the
10 ELISA technique. This sequence was identified by Proteus International UK, using
molecular techniques, as being a potentially useful peptide for production of anti-PrP
antibodies that could be used successfully for TSE detection. The highlighted sequence
is used to raise rabbit anti-PrP antibodies. The peptide (polypeptide, protein) is
conjugated to activated ovalbumin, BSA or KLH and injected intra-muscularly or
15 sub-cutaneously in Freund's Complete Adjuvant. Booster injections are sub-cutaneous
and Freund's Incomplete Adjuvant is used. The rabbits are bled at 30 and 50 days post
immunisation, or until a satisfactory titre is reached. Monoclonal antibodies can then be
produced if required.

20

2. Enfer Buffers 1-3 are produced and supplied by Enfer Scientific.

Homogenising Buffer 1: 16% Methanol/Ethanol
15 % Sodium Dodecyl Sulphate
25 0.1% Bovine Serum Albumin

The buffer is made up to the required volume, stirring in 0.1M TRIS/HCL Buffer, final
pH of 8.0+0.1. This buffer is stored from 16-25°C with an expiry of 1 year.

30 Differential Buffer 2: Proteinase K

This buffer is made up to the required concentration (0.1 to 10mg/ml) in 0.1M TRIS/HCL. Buffer, final pH of 8 + 0.1. This buffer is stored at -20°C with an expiry of 1 year.

- 5 Assay priming Buffer 3: 3M Guanidine or 6M Urea
 0.1M Sodium Hydroxide 0.1M Sodium Hydroxide

This buffer is made up to the required concentration in ROH₂O (reverse osmosis distilled water) and allowed to reach room temperature before checking pH, which
10 should preferably be greater than 11. This buffer is stored at 16-25°C with an expiry of 1 year.

3. Wash Buffers 1-2 are produced and supplied by Enfer Scientific.

- 15 Wash Buffer 1: 5% Sodium Chloride

This buffer is made up to the required volume in ROH₂O. pH irrelevant. This buffer
is stored at room temperature with an expiry of 1 year .

- 20 Wash Buffer 2: 0.1M Phosphate Buffered Saline
 0.5% Tween 20

This buffer is made up to the required volume in ROH₂O with a pH of 7.6 + 0.1.
This buffer is stored at room temperature with an expiry of 1 year.

25

4. Chemiluminescent reagent is supplied by Johnson and Johnson, Clinical
Diagnostics, UK and results are read using a Labsystems Chemiluminometer, available
from Medical Supply Company, Dublin, Ireland.

- 30 Determination of antibody concentration - Grid Assay

Negative and positive controls are put in duplicate into every second set of wells. A series of antibody dilutions are made up 1/500 to 1/2000 as shown in Table 1 and the test is run. At the primary antibody addition stage the dilutions of primary antibody are put onto the negative and positive sample wells. At the end of the test the antibody concentration which gives the highest positive control value, without giving a high negative control result is used.

Table 1

Primary Antibody Conc.		1	2	3	4	5	6	7	8	9	10	11	12
1/500	A	Neg											
	B	Pos											
1/1000	C	Neg											
	D	Pos											
1/1500	E	Neg											
	F	Pos											
1/2000	G	Neg											
	H	Pos											

10

Example 2

The antibody optimisation assay is carried out as follows: positive and negative tissue, confirmed by the Veterinary Research Laboratory is used. This is the same material used for controls in the assay.

The tissue is diluted 1/15 in Homogenising buffer and homogenised for 2 minutes. 200ul of negative homogenate is then placed in rows A, C, E and G. 200ul of positive homogenate is placed in rows B, D, F and H. The plate is centrifuged for 10 mins at 400 rpm.

20

20ul of differential buffer is added to the assay plate and 100ul of each sample is transferred to corresponding wells.

The plate is then sealed and incubated for 1 hour at 37°C,

The plate is washed with wash buffer 1 and 200ul of primary buffer is added. This is
5 incubated for 15 minutes at 37°C.

A series of antibody solutions are made up in wash buffer 2, i.e. 1/500, 1/1000, 1/1500 and 1/2000, if the antibody has a higher titre or lower titre than this range a higher or extended range can be tried after the initial assay.

10

Following incubation in priming buffer the plate is washed with wash buffer 2.

150ul of each antibody concentration is then added to the appropriate wells and incubated for 40 minutes at 37°C. The plate is washed with wash buffer 2 and 150ul of secondary antibody is added to all wells for 30 minutes at 37°C and then the plate is
15 washed again with wash buffer 2 and 150ul of chemiluminescence substrate is added to all wells. After a 3 minute incubation at 37°C the wells are read the chemiluminometer.

The antibody concentration which gives the best result i.e. low negative preferably <1.0, while giving a good positive result eg 500 is the concentration chosen.

20

As mentioned earlier a higher dilution range can be selected before the antibody is used, but this assay gives a good idea whether or not the antibody can be used.

1/500	{	N	4.235	3.897	3.554	4.116	2.698	3.487	4.679	3.229	4.569	3.487	3.778	4.946
	{	P	724.5	759.4	811.6	792.4	772.5	851.3	764.2	844.2	793.4	882.4	816.7	799.2
1/100	{	N	2.987	2.897	2.378	2.556	2.446	2.791	3.016	1.945	2.069	2.449	2.164	2.310
	{	P	563.4	547.2	538.0	587.1	564.2	603.7	589.4	556.1	567.3	613.7	508.6	543.7
1/500	{	N	1.579	0.945	1.344	1.067	1.337	1.298	1.049	0.879	1.346	1.222	1.340	1.206
	{	P	457.6	476.5	503.0	402.1	449.5	432.8	463.7	433.0	492.7	409.7	430.9	443.1
1/2000	{	N	0.861	0.936	0.727	0.914	0.866	0.569	0.612	0.493	0.721	0.642	0.463	0.762
	{	P	366.9	322.3	390.1	377.5	376.1	349.4	346.7	334.8	329.4	367.4	329.4	337.9

Example 3

Experimental Data on the suitability of the Enfer TSE assay as a means of detecting TSE in different tissues, namely, central nervous tissues (CNS), lymph tissue and blood
5 and in different species, ovine and bovine.

Results of routine testing using the TSE Assay of the invention in 2001.**Bovine CNS tissue**

10 Total samples tested : 664942
No. of positives detected : 134
No. of positives confirmed : 134
by immunohistochemistry

15 Ovine CNS tissue

Total samples tested : 16467
No. of positives detected : 60
No. of positives confirmed : 60
by immunohistochemistry

20

Ovine Lymph tissue

Total samples tested : 6974
No. of positives detected : 10
Confirmed : 9*
25 by immunohistochemistry

It was not possible to confirm one lymph tissue sample. This tissue was from a 14 week old lamb and was in the very early stages of infection. This may explain the difficulties with confirmation.

30

22

Ovine Blood tissue (1998)

Total tested : 3

No. of positives : 3

- 5 This was fresh blood derived from clinical Scrapie cases. All cases were found to be weakly positive and no confirmatory method exists to do a follow up analysis.

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CLAIMS

- 5 1. A method of detecting TSE comprising (a) treating a sample of tissue, blood or a blood derivative from a test subject with alcohol and a detergent, (b) adding an agent which degrades normal prion protein (c) adding an agent which denatures abnormal prion protein, (d) adding a prion.- specific antibody and (e) detecting binding of the antibody to the sample.
- 10 2. A method of fixing abnormal prion protein to a substrate comprising (a) treating the protein or a sample suspected to contain the abnormal prion protein with alcohol and a detergent and (b) adding a protease, in the presence of the substrate.
- 15 3. A method as claimed in claim 1 or 2 wherein step (a) further comprises the addition of a protein.
4. A method as claimed in claim 3 wherein the protein is a high molecular weight protein, preferably selected bovine serum albumin, ovalbumin, normal serum of animal
20 origin, an albumin substitute such as Prionex (trade mark), or derivatives of such.
5. A method as claimed in claim 3 or 4 wherein the protein is formulated in a buffer containing up to 5%, preferably 0.05 to 0.4% protein.
- 25 6. A method as claimed in any preceding claim wherein the alcohol used in step (a) is a C₁ to C₂₄ alcohol, preferably a C₁ to C₅ alcohol.
7. A method as claimed in claim 6 wherein the alcohol is selected from methanol, ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, nonanol, decanol,
30 undecanol, dodecanol, tridecanol, , pentadecanol, cetyl alcohol, heptadecanol, stearyl alcohol, nonadecanol, eicosanol, docasanol, dodecyl alcohol, lauryl alcohol,

octadecanol, and derivatives of each, including primary, secondary and tertiary derivatives, preferably methanol or ethanol.

8. A method as claimed in any preceding claim wherein the alcohol is formulated in a buffer containing up to 30% (by weight), preferably 10 to 20 more preferably 8 to 12% alcohol.

9. A method as claimed in any preceding claim wherein the anionic detergent of step (a) is selected from sodium dodecyl sulphate, sarcosyl, lithium dodecyl sulphate, cholic acid, deoxycholic acid, Triton X-100, octanoic acid, Brij (trade mark) 35 solution, N-Cetyl-N,N,N-trimethylammoniumbromide, CHAPS, CHAPSO, Digitonin, nonanoyl-N-methylglucamide, n-Octyl-B-D-glucopyranoside, alkyl sodium sulphate, dioctyl sodium sulfosuccinate, sodium oleate, sodium lauryl sulphate, 1,2,3-Octanetriol, 3-Diethylamino-1 propyne, Chondroitinase ABC, sodium Ionophore 1, and derivatives of each, most preferably sodium dodecyl sulphate.

15

10. A method as claimed in any preceding claim wherein the detergent is formulated in a buffer containing 5 to 30%, more preferably 10 to 20% detergent.

11. A method as claimed in any preceding claim wherein in step (b) the agent which degrades normal prion protein is a protease.

12. A method as claimed in claim 11 wherein the protease is a broad spectrum protease, preferably selected from proteinase K, subtilisin Carlsberg, pepsin and derivatives of each.

25

13. A method as claimed in wherein claim 11 wherein the protease is formulated in a buffer containing up to 10mg/ml, preferably 0.05mg/ml to 4mg/ml, more preferably 0.2mg/ml to 0.6mg/ml protein.

14. A method as claimed in any preceding claim wherein the agent which denatures abnormal prion protein in step (c) is a denaturing salt solution.

15. A method as claimed in claim 14 wherein the agent is selected from guanidine, urea, a standard denaturing solution e.g. Merck denaturing solution and derivatives of each.
- 5 16. A method as claimed in any preceding claim wherein the denaturing agent further includes up to 0.5M sodium hydroxide.
17. A method as claimed in any preceding claim wherein the denaturing salt is formulated in a buffer containing 0.1 to 10M, preferably 2 to 8M denaturing salt.
- 10 18. A method as claimed in any preceding claim wherein the buffer of step (a) (homogenising buffer) is made up to have a final pH of 5.0 to 11.0, preferably pH 7.0 to 9.0, more preferably pH 7.5 to 8.5.
- 15 19. A method as claimed in any preceding claim wherein the buffer of step (b) (differential buffer) is made up to a final pH of 2.0 to 11.0, preferably pH 2.0 to 8.5, more preferably 7.5 to 8.5.
- 20 20. A method as claimed in any preceding claim wherein the buffer of step (c) (assay priming buffer) has a pH of 1 to 14, preferably 9 to 12, more preferably at least 11.
- 25 21. A method as claimed in any preceding claim wherein following step (b) the reaction mixture is washed with a first wash buffer comprising up to 10%, preferably 4 to 6% buffered solution.
22. A method as claimed in any preceding claim wherein the assay priming buffer is allowed to incubate with the reaction mixture for about 5 to 30 minutes, preferably 10 to 20 minutes.
- 30 23. A method as claimed in any preceding claim wherein after treatment with the assay priming buffer the mixture is washed with a second wash buffer having a final pH of 5.0 to 11.0, preferably pH 7.0 to 8.0.

24. A method as claimed in claim 21 wherein the first wash buffer comprises sodium chloride, calcium chloride, zinc chloride.
25. A method as claimed in claim 23 wherein the second wash buffer comprises phosphate buffered saline with the optional addition of 0 to 5% Tween 20.
26. A method as claimed in any preceding claim wherein the blood or tissue sample is dispensed onto a support, suitably a microtitre tray.
27. A method as claimed in any preceding claim wherein the prion specific antibody is raised to a prion sequence which is to the 5' side of amino-acid number 60 of the prion sequence.
28. A test as claimed in any preceding claim comprising obtaining a sample of blood or a blood derivative or tissue from a test subject, reacting the tissue, blood or blood derivative sample with a prion specific antibody and detecting the binding of the antibody to the sample by standard immunoassay, wherein the tissue, blood or blood derivative sample is mixed prior to addition of the antibody with a first buffer comprising 0 to 30% of C₁ to C₅ alcohol, 5 to 30% sodium dodecyl sulphate, or 5 to 30% sodium dodecyl sulphate and 0 to 5% sarcosyl, and 0 to 5% bovine serum albumin made up to volume in about 0.1 M TRIS/HCL or phosphate buffer, the buffer having a final pH of 7.0 to 9.0.
29. A test for Transmissible Spongiform Encephalopathies comprising obtaining a sample of blood or blood derivative or tissue from a test subject, reacting the tissue, blood or blood derivative sample with a prion specific antibody and detecting the binding of the antibody to the sample by standard immunoassay, wherein the blood or tissue sample is dispensed onto an inert support and incubated with a buffer comprising up to 10 mg/ml, preferably 0.05mg/ml to 4mg/ml, more preferably 0.2mg/ml to 0.6mg/ml, of a broad spectrum protease enzyme in a buffer with a final pH of 2.0 to 11.0, preferably pH 2.0 to 8.5, more preferably 7.5 to 8.5, prior to reaction with the antibody.

30. A test for Transmissible Spongiform Encephalopathies comprising obtaining a sample of blood or a blood derivative or tissue from a test subject, reacting the tissue, blood or blood derivative sample with a prion specific antibody and detecting the
5 binding of the antibody to the sample by standard immunoassay, wherein the sample is incubated with a buffer comprising 0.1 to 10M denaturing solution, preferably 3 to 6M, more preferably 0.5 to 3M Guanidine (optimal range 2 to 4M) or 2 to 8M urea (optimal range 4 to 6M), and up to 0.5M, preferably 0.1 to 0.2M sodium hydroxide made up in reverse osmosis distilled water, with a pH of at least 11 at room temperature, prior to
10 reaction with the antibody.

31. A buffer selected from:

- (1). 0 to 30% of C₁ to C₂₄ alcohol (optimal range 10 to 20%) 5 to 30% detergent (optimal range 10 to 20%), and a 0 to 5% proteinase (optimal range 0.05 to 0.4%) made
15 up to volume in a buffer having a final pH of 5.0 to 11.0 (optimal range 7.5 to 8.5).
- (2). 0.05mg/ml to 4mg/ml (optimal range 0.2mg/ml to 0.6mg/ml) of a protease enzyme in a buffer having a final pH of 2.0 to 11.0 (optimal range 2.0 to 8.5).
- (3). 0.1 to 10M denaturing solution (optimal range 2 to 8M).

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WO 02/057789 A3

(54) Title: TEST FOR TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES

(57) Abstract: A method of detecting transmissible spongiform encephalopathies is described which involves (a) treating a sample of tissue, blood or a blood derivative from a test subject with alcohol and a detergent, (b) adding an agent which degrades normal prion protein (c) adding an agent which denatures abnormal prion protein, (d) adding a prion-specific antibody and (e) detecting binding of the antibody to the sample. Also described is a method of fixing abnormal prion protein to a substrate comprising (a) treating the protein or a sample suspected to contain the abnormal prion protein with alcohol and an anionic detergent and (b) adding a protease, in the presence of the substrate.

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International Application No

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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 35236 A (ENFER TECH LTD ;CONNOR MICHAEL O. (IE)) 13 August 1998 (1998-08-13) page 7, line 1 -page 15, line 10	1-31
X	DE 39 21 839 A (HENKEL KGAA) 17 January 1991 (1991-01-17) page 2, line 19-25, 51-53, 64-68	31
A	EP 0 861 900 A (ZUERICH ERZIEHUNGSDIREKTION) 2 September 1998 (1998-09-02) page 9, line 40 -page 10, line 25 page 11, line 21 -page 12, line 55 page 15, line 14-39 page 8, line 21-23	1-31

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☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 773 572 A (MEE ROGER PAUL ET AL) 30 June 1998 (1998-06-30) column 19, line 35-40 column 20, line 12-21 -----	1-31
A	US 5 633 349 A (REICHL HERWIG) 27 May 1997 (1997-05-27) column 4, line 47 -column 5, line 44; claims 1-11 -----	1-31

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IE 02/00006

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9835236	A	13-08-1998	AU 738606 B2	20-09-2001
			AU 6004698 A	26-08-1998
			EP 0909388 A2	21-04-1999
			WO 9835236 A2	13-08-1998
			NZ 333518 A	29-06-2001
			US 2001010918 A1	02-08-2001
DE 3921839	A	17-01-1991	DE 3921839 A1	17-01-1991
			AT 105583 T	15-05-1994
			DE 59005691 D1	16-06-1994
			DK 482046 T3	26-09-1994
			WO 9100333 A1	10-01-1991
			EP 0482046 A1	29-04-1992
			ES 2052262 T3	01-07-1994
			JP 2975428 B2	10-11-1999
			JP 4506475 T	12-11-1992
			PT 94540 A , B	20-03-1991
EP 0861900	A	02-09-1998	WO 9837210 A1	27-08-1998
			EP 0861900 A1	02-09-1998
			AU 742838 B2	10-01-2002
			AU 6498698 A	09-09-1998
			EP 1007700 A1	14-06-2000
			NZ 336656 A	31-05-2002
US 5773572	A	30-06-1998	AT 177754 T	15-04-1999
			AU 675053 B2	23-01-1997
			AU 3089292 A	28-06-1993
			CA 2124953 A1	10-06-1993
			DE 69228701 D1	22-04-1999
			DE 69228701 T2	29-07-1999
			DK 616613 T3	27-09-1999
			EP 0616613 A1	28-09-1994
			ES 2128362 T3	16-05-1999
			WO 9311155 A1	10-06-1993
			GR 3029740 T3	30-06-1999
			JP 7501798 T	23-02-1995
			NZ 246059 A	28-08-1995
			US 6379905 B1	30-04-2002
			ZA 9209392 A	27-07-1993
US 5633349	A	27-05-1997	AT 408191 B	25-09-2001
			AT 162991 A	15-05-1993
			AT 149355 T	15-03-1997
			DE 59208095 D1	10-04-1997
			DK 530173 T3	21-04-1997
			EP 0530173 A2	03-03-1993
			ES 2101077 T3	01-07-1997
			GR 3023206 T3	30-07-1997